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The Carbon Assimilation Pathways of *Methylococcus capsulatus*, *Pseudomonas methanica* and *Methylosinus trichosporium* (OB3B) during Growth on Methane

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D-*arabino*-3-Hexulose 6-phosphate was prepared by condensation of formaldehyde with ribulose 5-phosphate in the presence of 3-hexulose phosphate synthase from methane-grown *Methylococcus capsulatus*. The 3-hexulose phosphate was unstable in solutions of pH greater than 3, giving a mixture of products in which, after dephosphorylation, allulose and fructose were detected. A complete conversion of D-ribulose 5-phosphate and formaldehyde into D-fructose 6-phosphate was demonstrated in the presence of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase (prepared from methane-grown *M. capsulatus*). D-Allulose 6-phosphate was prepared from D-allose by way of D-allose 6-phosphate. No evidence was found for its metabolism by extracts of *M. capsulatus*, thus eliminating it as an intermediate in the carbon-assimilation process of this organism. A survey was made of the enzymes involved in the regeneration of pentose phosphate during C_1 assimilation via a modified pentose phosphate cycle. On the basis of the presence of the necessary enzymes, two alternative routes for cleavage of fructose 6-phosphate are suggested, one route involves fructose diphosphate aldolase and the other 6-phospho-2-keto-3-deoxygluconate aldolase. A detailed formulation of the complete ribulose monophosphate cycle of formaldehyde fixation is presented. The energy requirements for carbon assimilation by this cycle are compared with those for the serine pathway and the ribulose diphosphate cycle of carbon dioxide fixation. A cyclic scheme for oxidation of formaldehyde via 6-phosphogluconate is suggested.

Previous work led to the proposal that the net incorporation of C_1 units by *Methylococcus capsulatus* and *Pseudomonas methanica* during growth on methane or methanol proceeds by way of a modified pentose phosphate cycle (see Quayle, 1972). The essence of this cycle lies in the condensation of formaldehyde with a pentose phosphate to give a hexose phosphate which is isomerized to fructose 6-phosphate; subsequent metabolism of fructose 6-phosphate by reactions similar to those encountered in the ribulose diphosphate cycle then leads to regeneration of the pentose phosphate acceptor and net production of triose phosphate. Crude cell-free extracts of *P. methanica* and *M. capsulatus* catalysed the condensation of formaldehyde with D-ribose 5-phosphate to give the phosphate of a sugar tentatively identified as allulose (psicose) (Kemp & Quayle, 1966; Lawrence *et al.*, 1970). Subsequent work (Kemp, 1972, 1974) has shown that the sugar is not allulose but D-*erythro*-L-*glycero*-3-hexulose (D-*arabino*-3-hexulose) consistent with ribulose 5-phosphate being the acceptor molecule for formaldehyde rather than ribose 5-phosphate.

In the present paper, substrate quantities of the hexose phosphate product have been prepared through the use of 3-hexulose phosphate synthase (hexose phosphate synthase), solubilized and purified as described by Ferenci *et al.* (1974), and the properties of the product have been studied, including its isomerization to fructose 6-phosphate catalysed by phospho-3-hexuloisomerase. Authentic D-allulose 6-phosphate has been prepared and its reactions studied. No evidence has been found for its involvement in the carbon-assimilation pathway of the above organisms. An analysis has been made of the enzymes involved in the rearrangement of fructose 6-phosphate to ribulose 5-phosphate and triose phosphate and this has led to a detailed formulation of the ribulose monophosphate cycle of formaldehyde fixation.

Materials and Methods

D-Allulose (psicose) was obtained as 1,2:4,5 di-*O*-isopropylidene-D-allulose from the National Bureau of Standards, Washington, D.C., U.S.A., and hydrolysed to D-allulose as described by Tipson *et al.*, (1971). 1,2:5,6-Di-*O*-isopropylidene-D-glucose was purchased from Fluka A.G. Fluorochem Ltd., Glossop, Derby, SK13 9NU, U.K. Formaldehyde was prepared by heating 0.5 g of paraformaldehyde (BDH, Poole, Dorset, U.K.) in 5ml of water at 100°C in a

sealed tube for 15h. The strength of the resulting formaldehyde solution was determined by using alcohol dehydrogenase (EC 1.1.1.1) from yeast (Holzer & Goedde, 1965). Sedoheptulose 1,7-diphosphate, D-xylulose 5-phosphate and D-ribulose 5-phosphate as their respective sodium salts and transaldolase (EC 2.2.1.2) were purchased from Sigma (London) Chemical Co. Ltd., London, S.W.6, U.K. Phosphoriboisomerase (EC 5.3.1.6) was purchased from Calbiochem, London W.1, U.K. All the other sugar phosphates, coenzymes and enzymes were purchased from Boehringer Corporation, London W.5, U.K. The two enzymes, 3-hexulose phosphate synthase and phospho-3-hexuloisomerase, were purified from cell-free extracts of *M. capsulatus* as described by Ferenci *et al.* (1974), and the units of enzyme activity called are defined by Ferenci *et al.* (1974).

Preparation of buffers

Stock solutions of 0.2M-Na_2HPO_4 and 0.2M-KH_2PO_4 were mixed to give pH7.0. This solution was diluted to give a stock sodium–potassium phosphate buffer 0.1M with respect to phosphate (subsequently called sodium–potassium phosphate buffer).

Synthesis of D-*arabino*-3-*hexulose* 6-*phosphate* (D-*erythro*-L-*glycero*-3-*hexulose* 6-*phosphate*)

D-*arabino*-3-Hexulose 6-phosphate was prepared by the enzyme-catalysed condensation of formaldehyde with D-ribulose 5-phosphate by using 3-hexulose phosphate synthase: 1mmol of D-ribose 5-phosphate (sodium salt) was incubated with 120μg of phosphoriboisomerase in 98.5ml of 50mM-imidazole–HCl buffer, pH7.0, for 30min at 37°C. 3-Hexulose phosphate synthase (60units) was then added together with 120μg of phosphoriboisomerase, 0.5mmol of $MgCl_2$ and 1.5mmol of formaldehyde to a total volume of 100ml. After 10 and 20min, further 0.35mmol quantities of formaldehyde were added. The reaction was stopped after 30min, when 70% of the ribose 5-phosphate had disappeared, by precipitation of the sugar phosphates as barium salts as described by Horecker (1957). The barium salts of the sugar phosphates were converted into the sodium salts by dissolving in 2.5ml of 0.2M-H_2SO_4 and adding 0.5mmol of Na_2SO_4 and water to a total volume of 20ml. The resulting precipitate was collected by centrifugation at 38000*g* for 15min and washed twice with water, repeating the centrifugation each time. The supernatant and the washings were combined and the volume was adjusted to 50ml. A sample containing up to 500μmol of total sugar phosphates was taken, diluted to 50ml, and the pH adjusted to 8.0 with aq. NH_3. This solution was added, at a flow rate of 2ml/min, to a column (1.5cm × 5cm) of Dowex 1 (X8; Cl^- form) at 3°C and the sugar phosphates were eluted by using the borate complexing method of Khym & Cohn (1953). A linear gradient of 20–40mM-NH_4Cl in 0.04mM-$Na_2B_4O_7$, pH8.3 (total volume, 1litre) was used first, followed by 40mM-NH_4Cl in 0.01mM-$Na_2B_4O_7$, pH8.3, at a flow rate of 2ml/min. Fractions (10ml) were collected and D-ribulose 5-phosphate was eluted in the effluent volume between 400 and 700ml and the D-*arabino*-3-hexulose 6-phosphate in the effluent volume between 500 and 1200ml. The total yield in all fractions of the 3-hexulose phosphate was 70–80%; the best fractions from 710 to 1200ml (containing 40% yield) were pooled. After precipitation with $Ba(OH)_2$ and conversion of the sugar phosphates into the acid form by using Amberlite CG-120 (H^+ form), borate was removed by distillation under reduced pressure in a rotary evaporator (30°C) in the presence of methanol, as described by Zill *et al.* (1953). The evaporation was taken to near-dryness and the syrup was diluted with water to 50ml.

The pH was adjusted to 3.0 with NaOH and the D-*arabino*-3-hexulose 6-phosphate was stored at 3°C. Under these conditions, the 3-hexulose phosphate is stable for at least 3 months. The final yield from pentose phosphate was 25–30%. The dephosphorylated preparation gave a 3-hexulose/phosphate ratio of 1:0.97, and only one spot, which gave a pink colour with the spray for the ketose sugars, was found on paper chromatograms. The chromatographic properties of the 3-hexulose corresponded to those found by Kemp (1974).

Synthesis of D-*allulose* 6-*phosphate*

D-Allose was converted into D-allulose 6-phosphate by way of D-allose 6-phosphate as intermediate by the methods outlined below. In the preparation of D-allose 6-phosphate from D-allose advantage was taken of the slow phosphorylation of D-allose catalysed by yeast hexokinase (Sols *et al.*, 1958). D-Allose itself was synthesized from 1,2:5,6-di-*O*-isopropylidene-D-glucose by the method of Baker *et al.*, (1972).

D-Allose (2.5mmol), ATP (2.7mmol) and $MgCl_2$ (2.7mmol) were incubated with 2.5mg of hexokinase in 25ml of 0.1M-Tris–HCl buffer, pH8.0, at 37°C for 20h. The reaction was followed by assaying for ADP production (Adam, 1965) and the yield, on the basis of net ATP consumption was 95%. After incubation, the reaction mixture was heated at 95°C for 3min, filtered through Whatman filter paper no. 40, and diluted to 100ml with water. This solution was then added at a flow rate of 1ml/min to a column (1.5cm × 20cm) of Dowex 1 (X8; formate form). D-Allose 6-phosphate was eluted from the column by using a linear gradient from 0 to 1M-formic acid (total volume 2 litres) at a flow rate of 1ml/min, as described by Bartlett (1959). Fractions (10ml) were collected and samples were assayed for sugars by the anthrone method and for nucleotides by measuring E_{260}. D-Allose 6-phosphate was eluted from the column in the effluent

volume between 600 and 1000ml and the sugar phosphate was well separated from neutral sugars and nucleotides. The fractions containing D-allose 6-phosphate were pooled and evaporated under reduced pressure (30°C) to 200ml, after which the volume was adjusted to 400ml with water. The sugar phosphate was then precipitated as the barium salt. The yield of D-allose 6-phosphate from D-allose was 90%. The dephosphorylated sugar phosphate gave one brown spot on paper chromatograms when sprayed for aldose sugars, coincident with D-allose. The ratio D-allose/phosphate was 1:0.95.

D-Allose 6-phosphate was isomerized to D-allulose 6-phosphate with phosphoalloisomerase purified from *Klebsiella* (*Aerobacter*) *aerogenes* PRL R3 (a gift from Dr. F. J. Simpson, National Research Council of Canada, Halifax, Canada) grown on 0.1% D-allose as described by Matsushima & Simpson (1966). The purified phosphoalloisomerase catalysed the production from allose 6-phosphate of 1.25 μmol of allulose 6-phosphate/min per mg of protein. The assay conditions were as reported by Matsushima & Simpson (1966) except that authentic allulose was used in place of fructose as chromogenic standard. The sodium salt of D-allose 6-phosphate (300 μmol) was incubated with 6.4mg of purified phosphoalloisomerase in 600ml of 0.01 M-Tris–HCl buffer, pH 8.5, containing 0.01 M-cysteine–HCl, at 25°C. Equilibrium between D-allulose 6-phosphate (35%) and D-allose 6-phosphate (65%) was reached after 4h. The sugar phosphates were then precipitated as barium salts and converted into the sodium form. The borate-complexing method of Khym & Cohn (1953) was used to separate the two sugar phosphates. The sodium salts of the sugar phosphates in 100ml of water, adjusted to pH 8.0 with aq.NH_3, were added to a column (1.5cm × 20cm) of Dowex 1 (X8; Cl^- form) at a flow rate of 2ml/min, and the column was washed with 10ml of water. D-Allose 6-phosphate was eluted first with 1000ml of 1mM-$Na_2B_4O_7$–50mM-NH_4Cl, pH 8.3, at a flow rate of 2ml/min. The column was washed with 1000ml of 0.01mM-$Na_2B_4O_7$–25mM-NH_4Cl, pH 8.3, and then D-allulose 6-phosphate was eluted with 0.01 M-HCl in the effluent volume between 20 and 200ml, again at a flow rate of 2ml/min. The combined fractions containing D-allulose 6-phosphate were neutralized with NaOH and borate was removed from this sugar phosphate solution by the method of Khym *et al.* (1954), by applying the combined fractions to a column (1.5cm × 10cm) of Dowex 1 (X8; acetate form) and washing off borate with 1 litre of 0.01 M-ammonium acetate, pH 8.0, at a flow rate of 1 ml/min. The D-allulose 6-phosphate was eluted from the column with 1 M-ammonium acetate in the effluent volume between 40 and 120ml. The NH_4^+ was removed from the pooled fractions by passage through a column (2.5cm × 20cm) of Amberlite CG-120 (H^+ form) and the remaining acetic acid in the effluent was evaporated off under reduced pressure (30°C). The volume of the concentrated sugar phosphate solution was adjusted to 50ml with water and D-allulose 6-phosphate was precipitated as the barium salt. Total yield was 30% from D-allose 6-phosphate. The sugar obtained from dephosphorylating the sugar phosphate co-chromatographed with standard D-allulose and gave a spot of the same colour when sprayed for ketose sugars. The D-allulose/phosphate ratio was determined as 1:1.05.

Identification of sugar phosphates

Sugar phosphates were identified by dephosphorylation and paper chromatography of the resulting sugars. Approximately 10 μmol of the sugar phosphate to be identified was incubated for 30min at 37°C in 5ml of 20mM-Tris–HCl buffer, pH 8.5, containing 5 μmol of $MgCl_2$ and 20 μg of alkaline phosphatase (EC 3.1.3.1) from calf intestine. After incubation, the solution was heated for about 3min at 95°C, before removal of anions and cations by passing the mixture through a mixed-bed ion-exchange resin as described by Spiro (1966). The sugar solution was evaporated under reduced pressure to a thin syrup on a rotary evaporator at 40°C, and the volume was adjusted to 0.5ml with water. Samples of the sugars (0.05–3.0 μmol) were applied to paper chromatograms (Whatman no. 4; 46cm × 57cm), which were developed in two dimensions as described by Large *et al.* (1961). Aldose sugars were detected with a spray containing aniline (0.9ml) and phthalic acid (1.7g) in 100ml of water-saturated butan-1-ol, followed by heating at 105°C for about 10min; this gave a brown colour with aldohexoses (Partridge, 1949). Keto sugars were detected with a spray containing urea (3g), syrupy phosphoric acid (5ml) and ethanol (5ml) in 100ml of water-saturated butan-1-ol, followed by heating at 105°C for 10min, to give a blue-green colour for 2-hexuloses (Wise *et al.*, 1955). To identify an unknown sugar both its R_F value and colour of the spot were compared with standards.

Chemical determinations

Hexoses were determined by the anthrone method (Spiro, 1966) and with the *o*-aminodiphenyl reagent (Ashwell, 1966), 2-hexuloses and 2-pentuloses by the cysteine–carbazole method (Ashwell & Hickman, 1957) and aldopentoses by the phloroglucinol method (Ashwell, 1966). Specific standards for the determination of sugars and sugar phosphate esters were used, except when D-allose and D-allulose were used as standards in the determination of their respective sugar phosphates. Phosphate was assayed by the method of Fiske & SubbaRow (1925), borate by the method of Gupta & Boltz (1971), formaldehyde by the method of Nash (1953) and protein by the Folin–

Ciocalteu method with bovine serum albumin as standard (Lowry *et al.*, 1951).

Enzymic determinations

D-Glucose 6-phosphate was assayed with the enzyme D-glucose 6-phosphate dehydrogenase (EC 1.1.1.49), and D-fructose 6-phosphate was assayed by the same method in the presence of added phosphoglucoisomerase (EC 5.3.1.9) as described by Hohorst (1965*a*). D-*arabino*-3-Hexulose 6-phosphate was assayed by isomerization to D-fructose 6-phosphate with approx. 2 units of phospho-3-hexuloisomerase, and the D-fructose 6-phosphate formed was assayed as described above except that 1 mM-$MgCl_2$ was used instead of 5 mM-$MgCl_2$.

Growth of organisms

M. capsulatus (Foster & Davis, 1966) was grown at 37°C in an NH_4Cl–salts medium (Salem *et al.*, 1973) in a fermenter sparged with methane–air (1:20, v/v) and harvested as described by Lawrence *et al.* (1970). *P. methanica* (Dworkin & Foster, 1956) and *Methylosinus trichosporium* OB3B (Whittenbury *et al.*, 1970) were grown at 30°C in a KNO_3–salts medium (Leadbetter & Foster, 1958) gassed with methane–air as above.

Preparation of cell-free extracts

Freshly harvested cells were suspended in 4 vol. of 20 mM-sodium–potassium phosphate buffer, pH 7.0, and 5 mM-$MgCl_2$. *M. capsulatus* was disrupted by sonication for 6 min at 0–4°C in an M.S.E. 150W Ultrasonic disintegrator. *P. methanica* and *Mts. trichosporium* OB3B were disrupted by sonication for 4.5 min under the same conditions. Disrupted cells were centrifuged for 10 min at 6000*g* and the supernatants used as cell-free extracts.

Enzyme assays

Spectrophotometric assays were carried out in a Unicam SP. 1800 spectrophotometer at 30°C with freshly prepared cell-free extracts. 3-Hexulose phosphate synthase (D-*arabino*-3-hexulose 6-phosphate formaldehyde lyase) and phospho-3-hexuloisomerase (D-*arabino*-3-hexulose 6-phosphate 3,2-ketol-isomerase) were assayed as described by Ferenci *et al.*, (1974). Hydroxypyruvate reductase (D-glycerate–NAD^+ oxidoreductase, EC 1.1.1.29) was assayed at pH 7.5 as described by Large & Quayle (1963). Phosphoglucoisomerase (D-glucose 6-phosphate ketol-isomerase, EC 5.3.1.9) was assayed with D-fructose 6-phosphate (1 mM) as substrate in the reaction mixture described by Hohorst (1965*a*). Glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate–$NADP^+$ oxidoreductase, EC 1.1.1.49) was assayed with D-glucose 6-phosphate (1 mM) as substrate in the reaction mixture described by Hohorst (1965*a*). Phosphogluconate dehydrase (6-phospho-D-gluconate hydrolyase, EC 4.2.1.12) and phospho-2-keto-3-deoxygluconate aldolase (6-phospho-2-keto-3-deoxy-D-gluconate D-glyceraldehyde 3-phosphate lyase, EC 4.1.2.1.14) were assayed together with 6-phospho-D-gluconate as substrate by the method of Wood (1971). 6-Phosphogluconate dehydrogenase [6-phospho-D-gluconate–$NADP^+$ oxidoreductase (decarboxylating), EC 1.1.1.44] was assayed with 6-phospho-D-gluconate (1 mM) as substrate in the reaction mixture described by Hohorst (1965*b*). Phosphofructokinase (ATP–D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) was assayed as described by Sols & Salas (1966). Fructose diphosphate aldolase (D-fructose 1,6-diphosphate D-glyceraldehyde 3-phosphate lyase, EC 4.1.2.13) was assayed as described by Rutter & Hunsley (1966). Transaldolase (sedoheptulose 7-phosphate–D-glyceraldehyde 3-phosphate dihydroxyacetone transferase, EC 2.2.1.2) was assayed as described by Tchola & Horecker (1966). Transketolase (sedoheptulose 7-phosphate–D-glyceraldehyde 3-phosphate glycolaldehyde transferase, EC 2.2.1.1) was assayed with D-xylulose 5-phosphate (1 mM) and D-ribose 5-phosphate (1 mM) as substrates in the reaction mixture described by Racker (1965*c*).

Ribulose phosphate 3-epimerase (D-ribulose 5-phosphate 3-epimerase, EC 5.1.3.1) was assayed by incubating D-ribose 5-phosphate (3 mM) with 120 μg of phosphoriboisomerase per ml in the reaction mixture as described by Racker (1965*b*). The transketolase present in the extracts was used for the coupling of the reaction. Phosphoriboisomerase (D-ribose 5-phosphate ketol isomerase, EC 5.3.1.6) was assayed as described by Horecker *et al.* (1958). Fructose diphosphatase (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) was assayed with D-fructose 1,6-diphosphate (1 mM) as substrate; the formation of D-fructose 6-phosphate was continuously followed in the reaction mixture as described by Hohorst (1965*a*). Sedoheptulose diphosphatase (sedoheptulose 1,7-diphosphate 1-phosphohydrolase) was assayed with sedoheptulose 1,7-diphosphate (1 mM) as substrate; formation of sedoheptulose 7-phosphate was followed as described by Racker (1965*a*). Phosphoketolase [D-xylulose 5-phosphate D-glyceraldehyde 3-phosphate lyase (phosphate acetylating), EC 4.1.2.9] was measured by using D-xylulose 5-phosphate (1 mM) and D-fructose 6-phosphate (1 mM) as substrates in the reaction mixture described by Goldberg *et al.* (1966).

Results

Structure of D-*arabino*-3-*hexulose* 6-*phosphate*

The keto group of a ketose sugar or a derivative such as the sugar phosphate gives rise to an u.v.-absorption maximum at 280–290 nm (Swenson &

Barker, 1971). The value of the molar extinction coefficient (ε) at this absorption maximum is indicative of the extent to which the sugar is present in the acyclic keto form. This is illustrated in Fig. 1 for ribulose 5-phosphate ($\varepsilon_{285} = 62$ litre·mol^{-1}·cm^{-1}), which cannot form a ring structure, and fructose 6-phosphate ($\varepsilon_{290} = 5$ litre·mol^{-1}·cm^{-1}), which exists predominantly in a furanose ring structure (Swenson & Barker, 1971). The absorption spectrum of D-*arabino*-3-hexulose 6-phosphate, recorded in Fig. 1, shows a maximum at 285 nm ($\varepsilon = 70$ litre·mol^{-1}·cm^{-1}). This close similarity with ribulose 5-phosphate is consistent with the hexulose phophate possessing an acyclic structure in aqueous solution.

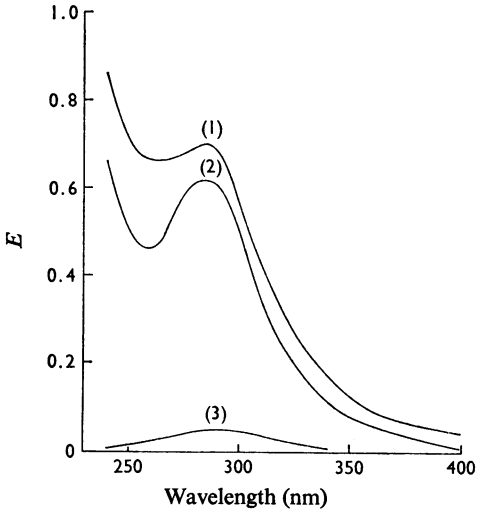


Fig. 1. *Absorption spectra of sugar phosphates*

The spectra of D-*arabino*-3-hexulose 6-phosphate (1), D-ribulose 5-phosphate (2) and D-fructose 6-phosphate (3) were recorded, against a blank of distilled water, for 10 mM solutions of their sodium salts in distilled water at pH 7.0 and 25°C using a Unicam SP. 1800 spectrophotometer (1 cm light-path).

Stability of D-*arabino*-3-*hexulose 6-phosphate in aqueous solution*

It is generally found that acyclic sugar phosphates in solution are more unstable than those forming a furanose or pyranose ring structure, more so in alkali than in acid (Pontis & Leloir, 1972). The stability of D-*arabino*-3-hexulose 6-phosphate has been examined and the results are shown in Table 1. The 3-hexulose phosphate was rapidly degraded at alkaline pH, and even at pH 7 and 20°C the half-life was only 40 h. Similar instability has been observed by Borenfreund & Dische (1957) for aqueous solutions of D-ribulose 5-phosphate and D-xylulose 5-phosphate.

Analysis of the solutions resulting from incubation of D-*arabino*-3-hexulose 6-phosphate at pH 5–11 showed fructose 6-phosphate to be present among the breakdown products in amounts up to 15% of the starting amount of 3-hexulose phosphate. The further possibility that 3-hexulose phosphate might also isomerize in part to allulose 6-phosphate was investigated in the following experiment.

A solution of D-arabino-3-hexulose 6-phosphate (10 μmol of the sodium salt, prepared as described in the Materials and Methods section, in 5 ml of water) was adjusted to pH 7.0 with NaOH. This solution was maintained for 48 h at 37°C, after which no 3-hexulose phosphate could be detected. Samples were then assayed by the cysteine–carbazole method for 2-hexuloses, 2-pentuloses and/or their phosphates, and enzymically for D-fructose 6-phosphate. The phosphate esters in the reaction mixture were dephosphorylated, desalted, and the resulting sugars were chromatographed. Samples corresponding to 1.5 ml of the initial 3-hexulose phosphate solution were applied to paper chromatograms, which were developed as described in the Materials and Methods section. Fructose and allulose were detected on the paper chromatograms tested for the presence of ketose sugars. The presence of fructose was consistent with the enzymic detection of fructose 6-phosphate in the reaction mixture; the presence of allulose suggests that allulose 6-phosphate was likewise

Table 1. *Stability of* D-*arabino*-3-*hexulose 6-phosphate*

Samples of D-*arabino*-3-hexulose 6-phosphate were diluted to a volume of 5 ml, after adjustment to the selected pH with HCl or NaOH, to give a final concentration of 2 mM. These solutions were stored at the temperatures shown, and 50 μl samples were removed at intervals and assayed for D-*arabino*-3-hexulose 6-phosphate as described in the Materials and Methods section. The stability of the 3-hexulose phosphate is shown as the half-life, i.e. the time in hours taken for 50% of the compound to disappear.

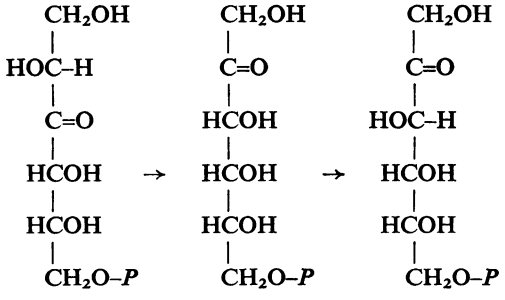
	Half-life of D-*arabino*-3-hexulose 6-phosphate (h)				
Temp. (°C)	pH ... 3	5	7	9	11
3	—	—	600	530	7
20	—	—	40	21	0.5
37	600	75	12	6	0.25

present in the same mixture. As only these two ketoses were detected in the reaction mixture, the amount of D-allulose 6-phosphate could be determined by the cysteine–carbazole method, after correcting for presence of D-fructose 6-phosphate which was determined enzymically. The results indicated that under the specified conditions the degradation of 10 μmol of D-*arabino*-3-hexulose 6-phosphate resulted in isomerization to 1.1 μmol of D-fructose 6-phosphate and 1.7 μmol of D-allulose 6-phosphate. Formaldehyde was also detected during the degradation of D-*arabino*-3-hexulose 6-phosphate, indicating that some aldol cleavage had taken place. However, no D-ribulose could be detected on paper chromatograms sprayed for ketose sugars in the above experiments. This might be explained in terms of the degradation of D-ribulose 5-phosphate itself under the conditions of incubation (Borenfreund & Dische, 1957).

The instability of D-*arabino*-3-hexulose 6-phosphate in aqueous solution may offer an explanation for the initial identification of the condensation product between pentose 5-phosphate and formaldehyde as D-allulose 6-phosphate (Kemp & Quayle, 1966). The present work has indicated that D-allulose 6-phosphate is formed non-enzymically from D-*arabino*-3-hexulose 6-phosphate in considerable amounts in aqueous solution even under conditions of neutral pH. It therefore seems likely that the original samples of the sugar phosphate product had partly isomerized to allulose 6-phosphate.

Possible role of D-*allulose 6-phosphate in the ribulose monophosphate cycle*

Although D-allulose 6-phosphate was thus ruled out as the condensation product between pentose 5-phosphate and formaldehyde, there remained the possibility that it might be involved as an intermediate between 3-hexulose 6-phosphate and D-fructose 6-phosphate in the subsequent reaction sequence of the ribulose monophosphate cycle, e.g.



D-*arabino*-3-Hexulose 6-phosphate — D-Allulose 6-phosphate — D-Fructose 6-phosphate

This possibility has been eliminated by studying the reactions of authentic D-allulose 6-phosphate (prepared as described in the Materials and Methods section) in the following experiments.

(1) D-Allulose 6-phosphate (4 μmol) was incubated with a mixture containing 10 μl of cell-free extract of *M. capsulatus* (140 μg of protein), 0.1 M-Tris–HCl buffer, pH 7.5, and 5 mM-$MgCl_2$, together with the reagent mixture for the D-fructose 6-phosphate assay in a total volume of 1 ml. The E_{340} was continuously recorded for 1 h at 30°C and over this period showed no change, indicating negligible formation of fructose 6-phosphate from allulose 6-phosphate.

(2) A mixture of D-allulose 6-phosphate (8 μmol), 0.1 M-Tris–HCl buffer, pH 7.5, 5 mM-$MgCl_2$ and 20 μl of cell-free extract (280 μg of protein) in a final volume of 2 ml was incubated for 1 h at 30°C. After this incubation period samples were assayed for the presence of aldohexoses with *o*-aminodiphenyl and aldopentoses with phloroglucinol as described in the Materials and Methods section. None was detected. The sugar phosphates in a further sample of the incubation mixture were dephosphorylated, desalted and the resulting free sugars were chromatographed (approx. 3 μmol of sugars were used for each paper chromatogram). When sprayed for ketose sugars only one blue-green spot appeared which co-chromatographed with D-allulose; no other spots were visible on the chromatograms sprayed for aldose sugars. This indicates that negligible conversion of allulose 6-phosphate to phosphates of other sugars was catalysed by extracts of *M. capsulatus*.

(3) An enzyme, phospho-3-hexuloisomerase, has been purified from cell-free extracts of *M. capsulatus*. This enzyme catalyses specifically the isomerization of D-*arabino*-3-hexulose 6-phosphate to D-fructose 6-phosphate, and D-allulose 6-phosphate is not a substrate for this enzyme (Ferenci *et al.*, 1974). There is thus no need to invoke an intermediary metabolite, such as allulose 6-phosphate, in the interconversion of 3-hexulose 6-phosphate to fructose 6-phosphate.

Conversion of D-*ribulose 5-phosphate and formaldehyde into* D-*fructose 6-phosphate*

The purification of the two enzymes, 3-hexulose phosphate synthase and phospho-3-hexuloisomerase (Ferenci *et al.*, 1974), has made it possible to demonstrate the quantitative enzymic conversion of D-ribulose 5-phosphate and formaldehyde into D-fructose 6-phosphate via D-*arabino*-3-hexulose 6-phosphate as intermediate (Table 2). In confirmation of the report of Kemp (1972), the commercial preparation of D-ribulose 5-phosphate was found to contain an uncharacterized inhibitor of 3-hexulose phosphate synthase which makes such preparations of D-ribulose 5-phosphate unsuitable for measurements of maximum rates of 3-hexulose phosphate synthase activity. D-Ribose 5-phosphate can also be converted completely into D-fructose 6-phosphate,

Table 2. *Conversion of* D-*ribulose 5-phosphate and formaldehyde into* D-*fructose 6-phosphate*

To the reaction mixture for the D-fructose 6-phosphate assay (see the Materials and Methods section) was added 0.2 μmol of formaldehyde together with either 0.1 μmol of D-ribulose 5-phosphate or 0.1 μmol of D-ribose 5-phosphate, in a total volume of 1 ml. Phosphoriboisomerase (120 μg), 3-hexulose phosphate synthase (5 units) and phospho-3-hexuloisomerase (2 units) were present as shown and formation of D-fructose 6-phosphate was followed at 340 nm in the presence (+) or absence (−) of the respective enzymes.

Sugar phosphate	Enzyme: Phosphoribo-isomerase	Enzyme: 3-Hexulose phosphate synthase	Enzyme: Phospho-3-hexuloisomerase	D-Fructose 6-phosphate formed from pentose phosphate (%)
D-Ribulose 5-phosphate	−	+	+	110
D-Ribulose 5-phosphate	−	−	−	0
D-Ribose 5-phosphate	+	+	+	97.5
D-Ribose 5-phosphate	+	−	−	0
D-Ribose 5-phosphate	−	+	+	0

Table 3. *Specific activities of enzymes in cell-free extracts of methane-grown bacteria*

The preparation of cell-free extracts and the assay procedures used are described in the Materials and Methods section. The specific activities are expressed as μmol/min per mg of protein. n.d., Not detected; —, not examined.

Enzyme	*M. capsulatus*	*P. methanica*	*Mts. trichosporium* (OB3B)
Hydroxypyruvate reductase	0.03	0.035	1.12
3-Hexulose phosphate synthase (hps)	2.3	1.9	n.d.
Phospho-3-hexulose isomerase (phi)	3.6	3.1	n.d.
Transaldolase (ta)	0.38	0.177	0.002
Transketolase (tk)	0.185	0.45	0.002
Ribulose 5-phosphate 3-epimerase (rpe)	0.13	0.235	—
Phosphoriboisomerase (pri)	1.5	1.07	0.03
Phosphofructokinase (pfk)	0.008	0.006	0.002
Fructose diphosphate aldolase (fda)	0.008	0.088	0.006
Phosphoglucoisomerase (pgi)	0.074	0.031	0.017
Glucose 6-phosphate dehydrogenase (gpd)	0.011	0.021	0.002
6-Phosphogluconate dehydrase/phospho-2-keto-3-deoxygluconate aldolase (edd)	0.026	0.053	n.d.
Fructose 1,6-diphosphatase	n.d.	0.075	0.02
Sedoheptulose 1,7-diphosphatase	n.d.	n.d.	n.d.
6-Phosphogluconate dehydrogenase (pgd)	0.015	0.013	n.d.
Phosphoketolase	n.d.	n.d.	n.d.

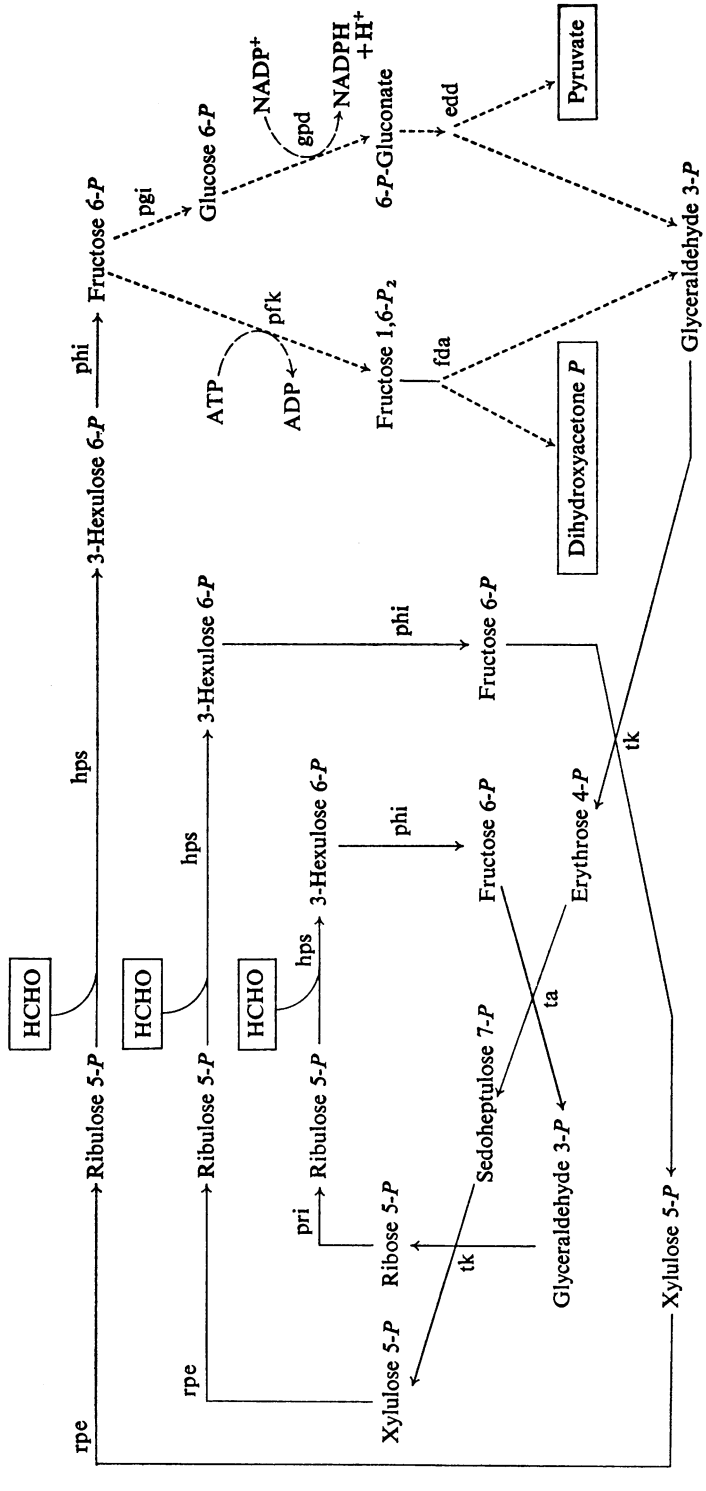
but only in the presence of added phosphoriboisomerase (Table 2). The reason for the complete conversion observed is that the equilibrium of the reaction catalysed by phospho-3-hexuloisomerase is very much in favour of D-fructose 6-phosphate formation (Ferenci *et al.*, 1974).

Activities of enzymes involved in C_1 metabolism

The regeneration of pentose phosphate during C_1 assimilation via 3-hexulose phosphate has been postulated to occur by way of a modified pentose phosphate cycle (Kemp & Quayle, 1967). The presence of all the enzymes necessary for the operation of the complete cycle has not hitherto been demonstrated. Table 3 presents such a survey for three methane-utilizing bacteria. *Mts. trichosporium* OB3B, which does not use the ribulose monophosphate pathway but assimilates methane by the serine pathway (Lawrence & Quayle, 1970), is included for comparison.

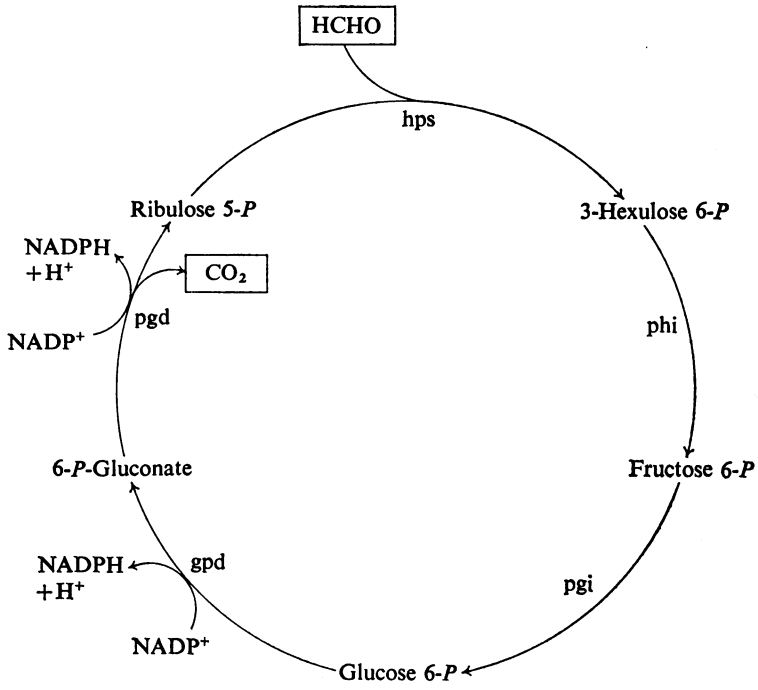
The rearrangement of two molecules of fructose 6-phosphate and one molecule of glyceraldehyde 3-phosphate to three molecules of ribulose 5-phosphate can be accomplished with transaldolase, transketolase, ribulose phosphate 3-epimerase and phosphoriboisomerase (Scheme 1). These four enzymes were found in high activity in cell-free extracts of *M. capsulatus* and *P. methanica*, but not in *Mts. trichosporium* OB3B.

The enzymic content of *M. capsulatus* and *P. methanica* suggested two alternative routes for effecting the necessary cleavage of fructose 6-phosphate to



Scheme 1. *Ribulose monophosphate cycle of formaldehyde fixation in M. capsulatus and P. methanica*

The key to the abbreviations used for the enzymes is given in Table 3.



Scheme 2. *Cyclic oxidation of formaldehyde*

The key to the abbreviations used for the enzymes is given in Table 3.

glyceraldehyde 3-phosphate (no evidence could be found for cleavage of fructose 6-phosphate by phosphoketolase): (*a*) cleavage by way of fructose diphosphate aldolase as in glycolysis; (*b*) cleavage by way of phospho-2-keto-3-deoxygluconate aldolase as in the Entner–Doudoroff pathway. Both these variants are shown in Scheme 1 and the entire complement of enzymes may then be assembled into the complete ribulose monophosphate cycle of formaldehyde fixation. It is not known whether one or both of the variants operates *in vivo*, although the fructose diphosphate aldolase pathway is energetically more favourable (see the Discussion section).

The presence of 6-phosphogluconate dehydrogenase in *M. capsulatus* and *P. methanica*, an enzyme also found in methane-utilizing bacteria by Davey *et al.* (1972), is interesting because this enzyme could complete a cycle for the complete oxidation of formaldehyde, shown in Scheme 2. It is not known whether this cycle actually operates *in vivo* but since there are no known NADP-linked enzymes that oxidize C_1 substrates in these organisms, it may be speculated that the cycle might serve as a source of NADPH for biosynthetic purposes.

Hydroxypyruvate reductase, a key enzyme of the serine pathway, has been found in *M. capsulatus* and *P. methanica*, but in much lower activity than in *Mts. trichosporium* OB3B. This fact, taken together with the finding of Salem *et al.* (1973) that another key enzyme of this pathway, malyl-CoA lyase, is present, suggests that a serine pathway may also operate in the first two organisms. A detailed analysis of all enzymes involved in the serine pathway is called for, particularly since Eccleston & Kelly (1973) have observed incorporation of formaldehyde into serine/glycine by whole cells of *M. capsulatus*. However, it is clear from the radioactive experiments of Kemp & Quayle (1966) that the serine pathway could only be of minor importance in carbon assimilation from methane and methanol.

Discussion

The work of Kemp (1972, 1974), Ferenci *et al.* (1974) and that described in the present paper has led to the modification of the cycle of formaldehyde fixation, first outlined by Kemp & Quayle (1967), to that now detailed in Scheme 1. One difference lies in the two-stage conversion of formaldehyde and pentose phosphate into fructose 6-phosphate. Initially it was thought to occur with D-ribose 5-phosphate as acceptor leading to D-allulose 6-phosphate as intermediate; the more recent work shows that D-ribulose 5-phosphate is the acceptor and this leads to D-*arabino*-3-hexulose 6-phosphate as intermediate. Tests with authentic D-allulose 6-phosphate have

failed to reveal any involvement of this compound in the metabolism of *M. capsulatus* even as an intermediate in the rearrangement of D-*arabino*-3-hexulose phosphate to fructose 6-phosphate. Another difference from the original scheme is an alternative route for cleavage of fructose 6-phosphate to triose phosphate.

It is interesting to compare the cycle with the related ribulose diphosphate cycle and pentose phosphate cycle. In the ribulose diphosphate cycle the rearrangement of five molecules of glyceraldehyde 3-phosphate to three molecules of pentose 5-phosphate is effected by way of the enzyme fructose diphosphate aldolase, fructose diphosphatase–sedoheptulose diphosphatase (one or two enzymes) and transketolase (pattern I). The rearrangement of two molecules of fructose 6-phosphate and one molecule of glyceraldehyde 3-phosphate to three molecules of pentose 5-phosphate can also be effected with transketolase and transaldolase (pattern II). Theoretically, either pattern of rearrangement could be utilized in the ribulose diphosphate cycle (Krebs & Kornberg, 1957) and the enzymic difference between the two patterns would lie in the involvement of fructose diphosphatase–sedoheptulose diphosphatase in one variant and transaldolase in the other. In the chloroplast it appears that pattern I is used for carbon dioxide fixation, transaldolase being used in the oxidative pentose phosphate cycle for the purpose of NADPH generation in the dark (Bassham, 1971). The high activity of transaldolase and low or undetectable activity of fructose diphosphatase–sedoheptulose diphosphatase in *M. capsulatus* and *P. methanica* (Table 2) suggests that in these organisms pattern II is used for the rearrangement reactions. There may be a biochemical rationale for this, stemming from the fact that both these organisms are obligate C_1-utilizers. Since they grow on no other substrate besides methane, methanol or dimethyl ether, they do not need to perform gluconeogenesis involving the action of fructose diphosphatase. Hence the choice of rearrangement pattern in their carbon-assimilation cycle in favour of one which does not involve fructose diphosphatase may perhaps be correlated with their peculiar nutritional properties. It will be of interest to see which pattern of rearrangement operates in any nutritionally versatile C_1-utilizing organisms which use the ribulose monophosphate cycle and are also capable of gluconeogenesis from C_3 intermediates.

The most extensive evidence for operation of the ribulose monophosphate cycle has been obtained in the case of *M. capsulatus* and *P. methanica*. Less extensive evidence indicates that the same or a closely similar cycle occurs in the following bacteria: *Methylomonas agile* Y1, *Methylomonas rosaceus* BG2, *Methylomonas methanica* 25, *Methylobacter capsulatus* 1521 and *Methylococcus minimus* TMC (Lawrence & Quayle, 1970); organism W1 (Dahl *et al.*, 1972); bacterium 4B6 (Colby & Zatman, 1972) and pseudomonad C (Stieglitz & Mateles, 1973). All these bacteria except pseudomonad C possess the peculiar property of being able to grow only on C_1 compounds or compounds such as dimethyl ether or dimethylamine in which individual C atoms are separated from each other by O or N atoms. Pseudomonad C is, however, nutritionally versatile. Hence the ribulose monophosphate cycle is not associated specifically with the obligate utilization of C_1 compounds. Evidence has also been obtained for occurrence of the cycle in the methanol-utilizing yeasts *Candida* N-16 (Fujii & Tonomura, 1973); *Kloeckera* sp. 2201, *Candida boidinii* sp. 0302 and *Pichia pastoris* (Diel *et al.*, 1974); *Candida boidinii* (Sahm & Wagner, 1974). Where tested, the specific activity of pentose phosphate-dependent fixation of formaldehyde catalysed by cell-free extracts of the yeasts appears to be lower than in bacteria.

There are now three pathways known in detail which can effect the net synthesis of a C_3 skeleton from C_1 compounds, namely the ribulose diphosphate cycle of carbon dioxide fixation, the serine pathway involving isocitrate lyase (Bellion & Hersh, 1972; Harder *et al.*, 1973; Cox & Zatman, 1973) and the ribulose monophosphate cycle of formaldehyde fixation. It is of interest to compare the input of energy necessary to make one molecule of pyruvate from three C_1 units in each of these cycles (see below). In each case the basic stoicheiometry of the individual cycle is calculated in terms of the production of the primary output molecule, e.g. glyceraldehyde 3-phosphate for the ribulose diphosphate cycle, and then a correction is made for converting this into pyruvate by established glycolytic steps. In this way the energy requirement of each cycle can be normalized to a common output molecule, pyruvate:

(1) Ribulose diphosphate cycle:

$$3CO_2 + 9ATP + 6NAD(P)H + 6H^+ + 5H_2O \rightarrow \text{glyceraldehyde 3-phosphate} + 9ADP + 6NAD(P)^+ + 8P_i$$

or

$$3CO_2 + 7ATP + 5NAD(P)H + 5H^+ + 5H_2O \rightarrow \text{pyruvate} + 7ADP + 5NAD(P)^+ + 7P_i$$

(2) Serine pathway:

$$2HCHO + CO_2 + 3ATP + 2NADH + 2H^+ + FP \rightarrow \text{phosphoglycerate} + 3ADP + 2P_i + 2NAD^+ + FPH_2$$

(FP = flavoprotein of succinate dehydrogenase)

or

$$2HCHO + CO_2 + 2ATP + 2NADH + 2H^+ + FP \rightarrow \text{pyruvate} + 2ADP + 2P_i + 2NAD^+ + FPH_2$$

If it is assumed that an organism can oxidize formaldehyde to carbon dioxide via nicotinamide nucleotide-linked dehydrogenases and thus gain two molecules of NADH, then the above equation can be modified to:

$$3HCHO + 2ATP + FP \rightarrow \text{pyruvate} + 2ADP + 2P_i + FPH_2$$

(3) Ribulose monophosphate cycle:

(a) Entner–Doudoroff variant

$$3HCHO + NADP^+ \rightarrow \text{pyruvate} + NADPH + H^+$$

(b) Fructose diphosphate aldolase variant

$$3HCHO + ATP \rightarrow \text{dihydroxyacetone phosphate} + ADP$$

or

$$3HCHO + ADP + P_i + NAD^+ \rightarrow \text{pyruvate} + ATP + NADH + H^+$$

Comparison of the above equations shows that the energy requirements for the different pathways decrease in the order $1 > 2 > 3(a) > 3(b)$. The fructose diphosphate aldolase variant of the ribulose monophosphate cycle actually yields ATP with respect to pyruvate synthesis and, if NADPH can be converted into ATP equivalents by oxidative phosphorylation, the Entner–Doudoroff variant would also be energy-yielding. It may therefore be predicted that the most efficient C_1-utilizing organisms will be found to utilize the ribulose monophosphate cycle.

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